PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
A61K 39/102, C07K 15/04
C12N 15/31

A1

(11) International Publication Number: WO 94/11024

(43) International Publication Date: 26 May 1994 (26.05.94)

(21) International Application Number: PCT/US93/10600

(22) International Filing Date: 5 November 1993 (05.11.93)

(30) Priority data:
07/973,070 6 November 1992 (06.11.92) US

(71) Applicant: REGENTS OF THE UNIVERSITY OF MINNESOTA [US/US]; Morrill Hall, 100 Church Street S.E., Minneapolis, MN 55455 (US).

(72) Inventors: CHOI, Keum, Hwa ; 1320 Coach Road, Apartment 135, St. Paul, MN 55108 (US). MAHESWARAN, Samuel, K. ; 1858 Dellwood Avenue North, Roseville, MN 55113 (US).

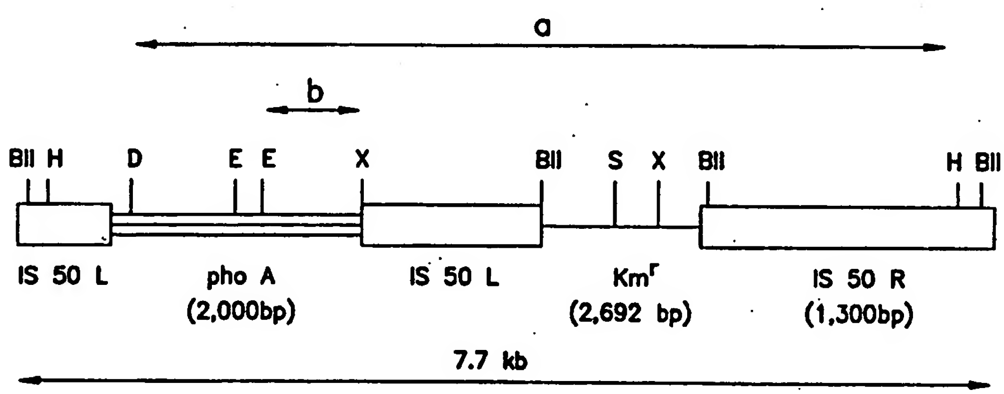
(74) Agent: BRUESS, Steven, C.; Merchant, Gould, Smith, Edell, Welter & Schmidt, 90 South Seventh Street, 3100 Norwest Center, Minneapolis, MN 55402 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: COMPOSITION PROTECTIVE AGAINST *P. MULTOCIDA* PASTEURELLOSIS INFECTION

RESTRICTION MAP OF TnphoA



a: 7kb PROBE DIGESTED WITH Dra I–Hpa I
b: 1.3 kb PROBE DIGESTED WITH EcoR I–Xho I

BII:Bgl II, H:Hpa I, D:Dra I, E:EcoR I, X:Xho I, S:Sma I

(57) Abstract

The invention provides vaccines and methods for protecting an animal against *P. multocida* associated pasteurellosis. A vaccine of the invention can be comprised of a stable avirulent immunogenic *P. multocida* mutant or a recombinantly produced *P. multocida* virulence factor. The avirulent immunogenic mutant can be a transposon-mediated mutant or a mutant having at least one genetically modified virulence gene. The methods of the invention include steps of producing an avirulent immunogenic mutant and administering an effective amount of the mutant to protect an animal against *P. multocida* pasteurellosis.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgystan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SI	Slovenia
CI	Côte d'Ivoire	KZ	Kazakhstan	SK	Slovakia
CM	Cameroon	LI	Liechtenstein	SN	Senegal
CN	China	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TG	Togo
CZ	Czech Republic	LV	Latvia	TJ	Tajikistan
DE	Germany	MC	Monaco	TT	Trinidad and Tobago
DK	Denmark	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	US	United States of America
FI	Finland	ML	Mali	UZ	Uzbekistan
FR	France	MN	Mongolia	VN	Viet Nam
GA	Gabon				

COMPOSITION PROTECTIVE AGAINST P. MULTOCIDA PASTEURELLOSIS INFECTION

Background of the Invention

Pasteurella multocida has been recognized as an important veterinary pathogen in disease processes of a variety of domestic and feral mammals and avian species. For example, P. multocida is associated with atrophic rhinitis and pneumonia of swine and with enzootic pneumonia in cattle. P. multocida is also the etiological agent of fowl cholera in avian species. P. multocida associated diseases cause major economic losses to the swine, cattle and avian industries.

Pasteurellosis or fowl cholera in turkeys is a highly contagious disease which occurs as a hyperacute, acute or chronic form. Pasteurella multocida belonging to different capsule types and somatic serotypes is the etiological agent of fowl cholera. The hyperacute and acute forms of the disease are characterized by septicemia, irreversible lesions in the lung, liver and spleen, and eventual death caused by the endotoxin of P. multocida. The chronic form is associated with high morbidity and the development of a carrier state.

The turkey industry in the United States is part of the $26 billion poultry industry and it produced 4.9 billion pounds of live weight in 1988 from 242.47 million turkeys, valued at $1.95 billion. The per capita consumption of turkey meat increased from 6.1 pounds in 1960 to 17.9 pounds in 1990. Death due to diseases has been a major cause of monetary loss to the U.S. turkey industry. In 1988 (the latest year for which statistics are available), disease cost the U.S. turkey industry an estimated $222 million, of which 50% was from respiratory diseases. The National Turkey Federation and the American Association of Avian Pathologists have recognized avian pasteurellosis, also known as fowl cholera, as one of the three most important diseases wreaking substantial economic losses to the U.S. turkey industry through increased mortality,

condemnation and medication costs. Carpenter et al., Avian Dis., 31:16-23 (1988).

With the recognition of the involvement of P. multocida in various animal diseases, efforts have been made to prevent this disease by vaccination with an array of commercial bacterins and attenuated live vaccines. Bierer et al., Poult. Sci., 51:408-416 (1972). However, efficacy and epidemiologic data available for these vaccines indicate that they are not totally effective in preventing the disease. It is now established that bacterins prepared from strains grown in vitro on artificial media, induce protection only against the somatic serotype from which the bacterin is made, i.e., serotype-specific immunity. Heddleston, Avian Dis., 6:315-321 (1962); Heddleston et al., Avian Dis., 14:626-635 (1970). The immunogen that is responsible for serotype-specific immunity has been identified as the lipopolysaccharide, while the immunogen which induce cross-protective immunity are the membrane associated proteins, called cross-protection factor (CPF) immunogens. Heddleston et al., Poult. Sci., 54:217-221 (1974).

Live vaccines induce cross-protective immunity against challenge exposure with multiple somatic serotypes of P. multocida. Bierer et al., cited supra. A serious disadvantage often encountered with the available live vaccines is that, in previously compromised turkeys, they actually cause systemic infection and death. Hofacre et al., National Turkey Federation Pasteurellosis Symposium at pages 12-16 (1989). Another important disadvantage has been the short duration of immunity induced by both bacterins and live vaccines. The protection never lasts beyond four weeks. In a recent symposium on fowl cholera disease sponsored by the National Turkey Federation, several speakers challenged the researchers to develop a new generation of superior vaccines that are safe and yet

still provide a broad spectrum of protection against all 16 somatic serotypes of P. multocida.

Thus, there is also a need for a vaccine specific for pasteurellosis that is simple to administer, yet provides long-lasting cross-protective immunity without adversely affecting the host. There is a need for a highly immunogenic avirulent live vaccine for fowl cholera which can be administered orally.

Summary of the Invention

The invention provides vaccines and methods for protecting an animal against P. multocida associated pasteurellosis. A vaccine of the invention can be comprised of an effective amount of a stable avirulent immunogenic P. multocida mutant or a recombinantly produced P. multocida virulence factor in a liquid non-toxic carrier. The avirulent immunogenic mutants can be a transposon-mediated mutant or a mutant having at least one genetically modified virulence gene located on a 9.4 kb EcorV fragment of the P. multocida genome. The methods of the invention include the steps of producing an avirulent immunogenic mutant and administering an effective amount of the mutant to protect an animal against pasteurellosis.

A transposon-mediated mutant can be a transposon insertion or deletion mutant. The mutant can be produced by introducing a transposon into the genome of a virulent strain of P. multocida under conditions favoring integration of the transposon. Suitable transposons include Tn1, Tn3, Tn5, TnphoA, Tn7, Tn9, Tn10, and functional fragments thereof. The transposon insertion mutants are selected for avirulence and the ability to provide immunity against pasteurellosis. Especially preferred mutants are those that provide long-lasting cross-protective immunity against pasteurellosis.

An avirulent mutant having a genetically modified virulence gene located on a 9.4 kb EcorV fragment of the P. multocida genome can be produced by standard methods of mutagenesis. The virulence gene can be genetically modified by transposon insertion or deletion mutagenesis, chemical mutagenesis, restriction endonuclease and exonuclease mutagenesis, and polymerase chain reaction mediated mutagenesis. The mutants so produced can then be selected for avirulence and protection against pasteurellosis. The genetic modification to a virulence gene located on a 9 kb EcorV fragment in the selected mutants can be verified by standard methods, such as restriction enzyme mapping.

A gene encoding a virulence factor on a 9.4 kb EcorV fragment can be subcloned and transformed into a suitable host so that a recombinant virulence factor can be produced. The virulence gene is subcloned under appropriate transcriptional and translational control regions to provide a high level expression of the virulence factor. The virulence factor can be identified and purified by standard methods. The virulence factor can then be used to immunize animals and provide protection against pasteurellosis.

The method of the invention provides for administering an effective amount of the avirulent immunogenic P. multocida mutant to an animal to provide protection against pasteurellosis. The mutant can be administered by several routes, including the parenteral route, nasal drops, aerosol, and preferably in the drinking water. The effective amount is that amount of the mutant that provides for protection against pasteurellosis, and preferably is about 10^8 CFU/ml to about 10^9 CFU/ml. A wide variety of animals can be immunized in the method of the invention including cattle, pigs, ducks, turkeys, and chickens. The preferred animal is the turkey.

Brief Description of the Figures

FIGURE 1 shows a restriction enzyme map of TnphoA.

FIGURE 2 shows Southern blot analysis of DNA digests of avirulent transposon mediated mutants of P. multocida.

Detailed Description of the Invention

The invention provides vaccines and methods for protecting animals against pasteurellosis including fowl cholera. A vaccine is comprised of avirulent immunogenic mutants of P. multocida that can provide immunity against P. multocida associated pasteurellosis. The vaccine can also be comprised of a recombinantly produced P. multocida factor, and preferably the virulence factor is a gene product encoded on a 9.4 kb EcorV fragment of the genome P. multocida. Once the avirulent mutant or recombinant virulence factor is produced, an effective amount of the vaccine is administered to the animal to provide for immunity against pasteurellosis including fowl cholera.

A. Vaccines

An immunogenic bacterium employed as the active component of a vaccine is a stable live avirulent immunogenic mutant of Pasteurella multocida that provides immunity against P. multocida. The mutant can be administered to an animal without causing disease or death and preferably provides long-lasting cross-protective immunity. The immunogenic bacteria can be a transposon-mediated mutant or a mutant having at least one genetically modified virulence gene located on a 9.4 kb EcorV fragment of the P. multocida genome. An effective amount of the immunogenic avirulent mutant bacteria or the recombinant virulence factor of the invention is combined with a physiologically acceptable non-toxic liquid vehicle to form the vaccine.

As used herein, "stable" means that the mutant maintains the desired characteristics for multiple passages through an animal or for multiple generations of growth. Preferably, the mutant has a reversion frequency of less than about 10^{-5} to about 10^{-10}, and more preferably less than about 10^{-6} to about 10^{-8}.

As used herein, "cross-protective immunity" refers to the capacity of the avirulent immunogenic mutant to protect the immunized animal from infection by multiple virulent serotypes of P. multocida, and preferably the immunogenic mutant protects against all virulent serotypes.

As used herein, "long-lasting immunity" refers to the capability of the immunogenic mutant to generate an immune response, preferably that lasts from at least about 6 weeks to about 20 weeks, and more preferably for the lifetime of the animal.

As used herein, "an effective amount" is the amount of immunogenic avirulent mutant or virulence factor that provides protection of the immunized animal against P. multocida associated pasteurellosis.

As used herein, a "transposon" refers to a DNA sequence that can move from place to place in a genome by processes which do not require extensive DNA sequence homology between the transposon and the site of insertion nor the recombination enzymes need for classical homologous crossing over.

An immunogenic avirulent mutant bacteria can be a transposon-mediated mutant. The transposon-mediated mutants are those mutants in which a transposon has been inserted or deleted from the genome of a virulent strain of P. multocida. A transposon insertion mutant is a mutant that has at least one transposon or a functional fragment thereof inserted in the genome at one or multiple sites. Preferably, the transposon inserts randomly in the genome. Transposon insertion mutants are then selected for the presence of transposon encoded

genes. The transposon insertion mutants can then be further selected for avirulence and for providing immunity against P. multocida associated pasteurellosis in animals. A transposon deletion mutant can be produced from avirulent transposon insertion mutants by selecting for mutants that have lost the transposon encoded genes but still maintain avirulence and the ability to protect animals against P. multocida associated pasteurellosis.

The transposon-mediated mutants can be produced by introduction of a transposon or functional fragment thereof into P. multocida and selecting for avirulent transposon insertion mutants. Suitable transposons are those that encode a marker gene including Tn1, Tn3, Tn5, TnphoA, Tn7, Tn9, and Tn10 and functional fragments thereof. The especially preferred transposon is TnphoA.

An avirulent immunogenic mutant can also be a mutant having at least one genetically modified virulence gene located on a 9.4 kb EcorV fragment of the P. multocida genome. Virulence genes can be identified and mapped by transposon-mediated mutagenesis. A virulence gene is one that is essentially nonfunctional or produces an essentially nonfunctional gene product in an avirulent mutant but is functional in a virulent P. multocida strain. An essentially nonfunctional gene can be one that is not expressed at a level sufficient to provide the gene-associated function, including virulence, to the mutant and/or one which is expressed but produces a nonfunctional gene product. An essentially nonfunctional virulence gene can be identified by assaying for function, including virulence of the gene product, and preferably a gene product having at least about 10- to about 1000-fold reduction in function is essentially nonfunctional. Alternatively, the gene product encoded by the essentially nonfunctional virulence gene can be identified by a change in physical characteristics of

the gene product including molecular weight, isoelectric point, and amino acid composition. A preferred mutant is one that has an essentially nonfunctional virulence gene encoded on a 9 kb EcorV fragment of the P. multocida genome.

Once identified, virulence genes in virulent strain of P. multocida can be rendered nonfunctional by mutations or genetic modifications generated by standard methods known to those of skill in the art, including transposon-mediated mutagenesis, chemical mutagenesis, restriction enzyme and/or exonuclease-mediated mutagenesis, and the like. The P. multocida mutants having at least one genetically modified virulence gene are selected by screening for conversion of the virulent strain of P. multocida into an avirulent strain and for the ability to protect against pasteurellosis in animals.

Specific examples of the avirulent mutants of the invention include the avirulent transposon insertion P. multocida mutants designated PmTn-294 and PmTn-396. Both mutants are characterized by expression of alkaline phosphatase activity, loss of resistance to complement mediated killing, and loss of virulence in turkeys. Preferred mutants of the invention include a mutant having the characteristics of ATCC No. 55394, deposited with the American Type Culture Collection, Rockville, Maryland, on February 17, 1993, and a mutant having the characteristics of ATCC No. 55395, deposited with the American Type Culture Collection, Rockville, Maryland, on February 17, 1993.

A vaccine of the invention can also be comprised of an effective amount of at least one recombinantly produced virulence factor from P. multocida in a liquid non-toxic vehicle. A virulence factor can be a gene product that is essentially nonfunctional in avirulent bacterial strain and functional in a virulent P. multocida strain. The

virulence factor can be identified as the gene product of a virulence gene of P. multocida by methods known to those of skill in the art including in vitro transcription translation systems. Alternatively, the virulence factor can be identified by a functional assay including virulence or by the ability to produce symptoms and lesions of the disease. A virulence factor that is essentially nonfunctional in avirulent mutant bacteria has at least about a 10- to 1000-fold reduction in functional activity. Optionally, the virulence factor can be identified by a change in its physical characteristics when the same factor is compared between virulent and avirulent mutants. To produce the recombinant virulence factor, a virulence gene is cloned from virulent P. multocida strains into an appropriate host organism by standard methods, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, NY (1989), and the recombinantly produced virulence factor is expressed. The virulence factor is also preferably selected for long-lasting cross-protective immunity against pasteurellosis in an animal. An effective amount of the recombinant virulence factor is an amount sufficient to provide for protection against pasteurellosis, preferably about 5 mg/kg to about 10 mg/kg. A preferred recombinantly produced virulence factor is a gene product encoded by a gene on a 9.4 kb EcorV fragment of the P. multocida genome.

To use the mutants of the present invention as a vaccine, cells of the mutant are combined with a suitable physiologically acceptable non-toxic liquid vehicle. Specific examples of suitable liquid non-toxic vehicles include buffered salt solutions, 0.85% saline and, preferably, drinking water. The amount of cells included in a given unit dosage form of the vaccine can vary widely and depends upon factors such as age, weight and physical condition of the animal. Such factors can

be readily determined by the clinician or veterinarian employing animal models or other test systems which are well known to those of skill in the art. Preferably, an effective amount of the mutant will range from about 1×10^6 to 1×10^{11} cfu/ml, and more preferably about 1×10^7 to 1×10^{10} cfu/ml. A unit dose of the vaccine can be administered parenterally, e.g., by subcutaneous or intramuscular injection, however oral or aerosol delivery is preferred. The preferred vaccine can be administered by mixing the mutant in the drinking water and making the water available to the animals. Alternatively, the vaccine can be administered intranasally by dropping into the nares or by aerosol. In a preferred version, a vaccine comprised of 10^7 cfu/ml of a mutant having the characteristics of ATCC No. 55394 or ATCC No. 55395 is administered in the drinking water to turkeys.

The vaccines can be administered to a variety of animals including cattle, pigs, ducks, turkeys, and chickens to protect against pasteurellosis. The especially preferred animal is the turkey.

B. Methods of Producing an Avirulent Transposon of P. Multocida and Immunizing an Animal Against Pasteurellosis

The invention also provides a method of immunizing an animal against pasteurellosis with a stable avirulent immunogenic transposon-mediated mutant of P. multocida. The method involves the steps of producing a stable avirulent immunogenic transposon-mediated mutant and administering an effective amount of the mutant to the animal to provide immunity against P. multocida associated pasteurellosis.

The preferred stable avirulent immunogenic transposon-mediated mutants of P. multocida can be produced by transposon-mediated mutagenesis. Transposon-mediated mutants include both those that have a transposon inserted into the bacterial genome, known

as insertion mutants, and those where the transposon has been inserted and then excised with a portion of the bacterial gene, creating a nonreverting deletion mutant. Transposon-mediated mutants are then selected for avirulence and for the ability to protect against pasteurellosis, and preferably for stability.

A transposon insertion mutant of P. multocida can be produced by standard methods known to those of skill in the art and as described by Taylor et al., J. Bacteriology, 171:1870 (1989). Briefly, a transposon in a suitable vector is introduced into P. multocida, preferably a virulent strain, under conditions that favor insertion of the transposon into the genome of the bacteria. For example, a transposon can be placed in a suicide vector. A suicide vector is one that can be introduced into a wide variety of bacteria but is only capable of replicating in certain types of bacteria. The inability of the suicide vector to replicate favors selection of bacteria having the transposon inserted into the genome. The vector is preferably introduced into the P. multocida by transconjugation but can also be introduced by other methods known to those of skill in the art, such as electroporation or calcium phosphate precipitation. Once introduced into P. multocida, transposon insertion mutants are selected by the presence of transposon encoded marker genes and further selected for avirulence for animals, for protection against pasteurellosis and stability.

As used herein, a "transposon" is a DNA segment that can move to new locations in DNA molecules by processes which do not require extensive DNA sequence homology or recombination enzymes. Transposons can include marker genes encoding antibiotic resistance and transposition enzymes, and are typically bounded by a region of DNA sequences, known as insertion sequences, that mediate insertion of the transposon into DNA. For

example, the DNA sequences at the termini of insertion sequence 50 of the Tn5 transposon are:

5′ CTGACTCTTATACACAAGTAGCGTCCTGAACG. . .
3′ GACTGAGAATATGTGTTCATCGCAGGACTTGC. . .

. . .GCGCAGGGGATCAAGATCTGATCAAGAGACAG (SEQ ID NO:1)
. . .CGCGTCCCCTAGTTCTAGACTAGTTCTCTGTC

as reported by Berg et al., Biotechnology, 1(5):417 (July 1983). Transposons can be modified by methods known to those of skill in the art, as long as they retain the functional ability to insert into DNA. Some modified transposons are known as insertion sequences. All or portions of transposons can insert into one or more locations in a bacterial genome and, if they insert into a gene, typically form a mutant no longer having the function associated with that gene. Suitable transposons include Tn1, Tn3, Tn5, TnphoA, Tn7, Tn9, Tn10, and functional fragments thereof. The especially preferred transposon is the TnphoA transposon, which contains the left insertion sequence of Tn5 linked to the gene encoding a marker gene, such as the gene for alkaline phosphatase, and an antibiotic resistance gene, such as kanamycin resistance and tetracycline resistance.

Suitable vectors for introducing the transposon into P. multocida are vectors that favor integration of the transposon into the bacterial genome. Specific examples include suicide plasmids that can conjugate with but cannot replicate in P. multocida. Alternatively, P. multocida can be co-transformed with a plasmid containing the transposon and a plasmid of the same incompatibility group so that the plasmids will not be able to replicate in the cell. The preferred plasmid is a suicide vector such as pRT733 which can

transconjugate but not replicate in P. multocida and carries the TnphoA transposon.

P. multocida mutants with integrated transposons are selected by identifying those bacteria having at least one selectable marker gene encoded by the transposon. The selectable marker gene can include antibiotic resistance genes, such as kanamycin resistance genes, tetracycline resistance genes, and the like. Other marker genes can include reporter genes, such as the chloramphenicol acetyltransferase gene, the alkaline phosphatase gene, the β-galactosidase gene, and the like. The especially preferred marker gene is the alkaline phosphatase gene because this marker gene provides for selection of mutants having modified genes encoding membrane or secreted membranes. Alkaline phosphatase is detected as a secreted enzyme and it is believed that mutants secreting alkaline phosphatase have the transposon inserted into a gene that encodes a membrane or secreted gene product.

The transposon insertion mutants are further selected for avirulence. The avirulent mutants can be identified by either in vitro or in vivo methods. Avirulent mutants can be identified by in vitro assays that correlate with the in vivo virulence. A suitable example includes a complement-mediated lysis assay; virulent strains of P. multocida are resistant to complement-mediated lysis, whereas avirulent strains are susceptible to complement-mediated lysis. Alternatively, avirulent mutants can be identified and selected by the inability to cause death or disease in the animal by standard methods.

Avirulent mutants of the invention can be further selected for the ability to protect animals against pasteurellosis. Different amounts of an avirulent mutant can be administered to the animal. After about two weeks, the animals can be examined for the presence of protective immunity specific for P.

multocida by standard methods, including detecting antibodies by the ELISA test. The animals can also be challenged with at least one virulent strain of P. multocida. The avirulent mutants that protect against pasteurellosis caused by virulent P. multocida can be identified as well as the effective amount of the mutant providing protection against the disease. Protection against pasteurellosis can be determined by comparing the percentage of nonimmunized animals which die or show symptoms of the disease after challenge with those that were immunized with the avirulent mutant. Symptoms of the diseases associated with P. multocida in each species of animal are well known to those of skill in the art. An avirulent mutant that protects about 90-100% of animals from death or the symptoms of the disease is preferred.

An especially preferred avirulent mutant is one that provides long-lasting cross-protective immunity against pasteurellosis. An avirulent mutant can be selected for providing cross-protective immunity by challenging animals immunized with the avirulent mutant with all of the virulent serotypes and identifying avirulent mutants that provide protection against some or all of the virulent serotypes of P. multocida. Long-lasting immunity can be evaluated by challenging the animals immunized with the avirulent mutants after different time periods, from about 2 weeks to about 20 weeks. The preferred avirulent mutants can provide immunity against pasteurellosis from at least about 6 weeks up to about 20 weeks, and the especially preferred mutants provide lifetime immunity against pasteurellosis for the animal.

Preferably, the avirulent mutant of P. multocida is also a stable mutant. Stable mutants can be identified by growing the mutant for about 10 to 50 generations without the loss of desireable characteristics, such as avirulence and protection

against pasteurellosis. Reversion frequency can also be measured to determine stability by standard methods known to those of skill in the art. A stable avirulent mutant of the invention preferably has a reversion frequency of less than 10^{-5} to 10^{-10} and more preferably of about than 10^{-6} to about 10^{-8}. Alternatively, the mutant can be passed through animals for about 10 to 20 passages and examined for the rate of the loss of the desired characteristics of avirulence and protection against pasteurellosis. A stable mutant is one that can be passed through animals for about 10 to 20 passages and still maintain the desired characteristics.

The transposon-mediated mutant of the invention can also be a transposon-mediated deletion mutant. A transposon-mediated deletion mutant can be selected and isolated from the transposon insertion mutants produced and selected as described above. An avirulent transposon insertion mutant can be grown under conditions no longer selecting for the marker gene encoded by the transposon, such as a gene for antibiotic resistance or for alkaline phosphatase. Bacteria which have lost these marker genes can be further screened for maintenance of the avirulence characteristic. It is believed that, at a very low frequency of less than 10^{-8}, the transposon can excise from the genome of the bacteria and, if that excision is not perfect, can carry some of the DNA sequence from the gene into which the transposon initially inserted. When the transposon excises in this manner, a transposon-mediated deletion mutant is created. Transposon-deletion mutants can be identified and isolated by screening for those mutants that have lost the marker gene encoded by the transposon while still maintaining the avirulence characteristic. Once identified, the transposon deletion mutants can be further selected for stability and for providing long-lasting cross-protective immunity against pasteurellosis, as described above.

In a preferred version, a transposon-mediated mutant of P. multocida is produced. A suicide vector encoding the TnphoA transposon is introduced into a virulent strain of P. multocida by transconjugation. Transconjugates with the TnphoA transposon inserted into the genome are first selected by screening for antibiotic resistance and for secretion of alkaline phosphatase. Mutants that are resistant to antibiotics and which secrete alkaline phosphatase are then screened for avirulence in vivo and in vitro. An avirulent mutant which secretes alkaline phosphatase, isolated as described herein, has been deposited with the ATCC on February 17, 1993 and given Accession No. 55394. Another avirulent mutant which secretes alkaline phosphatase, isolated as described herein, has been deposited with the ATCC on February 17, 1993 and given Accession No. 55395. The mutant is then screened for the ability to provide protection against pasteurellosis in an animal. The avirulent mutant also preferably is stable and provides long-lasting cross-protective immunity against pasteurellosis.

Once produced, the transposon-mediated mutants of the invention are administered to the animal. Administration can occur by any one of several routes including parenteral,nasal drops, aerosol, and/or through the drinking water. An effective dose of each mutant can be determined as described above, but preferably is about 10^8 to 10^9 CFU/ml. The mutants can be administered to a variety of animal species including cattle, pigs, ducks, chickens, and turkeys but is preferably administered to turkeys.

C. Method of Producing an Avirulent Immunogenic Mutant Having at Least One Genetically Modified Virulence Gene And Immunizing Animals Against Pasteurellosis

The invention provides a method of immunizing an animal against P. multocida associated pasteurellosis with a stable avirulent immunogenic mutant of P. multocida wherein the mutant has at least one genetically modified virulence gene located in a 9.4 kb EcorV fragment of the P. multocida genome. The method involves the steps of producing a stable avirulent immunogenic mutant having a genetically modified virulence gene and administering an effective amount to an animal to provide immunity against pasteurellosis.

The avirulent immunogenic mutants can be produced by first identifying virulence genes and then genetically modifying the virulence genes located in a 9.4 kb EcorV fragment of the P. multocida genome. Virulence genes of P. multocida can be identified by using transposon insertion mutants to identify and to map the location of P. multocida virulence genes. It is believed that insertion of a transposon into a gene can result in inactivation of the gene. Transposon insertion mutants showing a loss of virulence have transposons inserted in genes required for virulence. The location of a transposon insertion in avirulent mutants can be detected and mapped by standard methods including Southern blot hybridization and DNA sequencing.

Once identified and mapped, the virulence genes can be genetically modified by standard methods known to those of skill in the art. A virulence gene is genetically modified resulting in the avirulence phenotype of the mutant. The virulence gene can be genetically modified so that the gene is expressed at a level below that required to produce virulence or it can be modified to produce an essentially nonfunctional gene product.

A genetically modified virulence gene can be produced by standard methods of mutagenesis. Suitable methods include transposon-mediated mutagenesis (insertion or deletion), chemical mutagenesis, restriction enzyme or exonuclease mutagenesis, and polymerase chain reaction mediated mutagenesis. The preferred method for generating the mutants is by transposon-mediated mutagenesis. A preferred mutant of the invention is a mutant having a genetically modified virulence gene located in a 9.4 kb EcorV fragment of the P. multocida genome.

A genetically modified virulence gene can be detected by a variety of methods known to those of skill in the art. The genetic modification can be detected by a change in restriction enzyme mapping, ribosomal RNA profile, or by a change detected by direct DNA sequencing. Alternatively, the genetically modified virulence gene can be detected by a functional assay for the virulence gene product. A genetically modified virulence gene preferably expresses a gene product that is essentially nonfunctional in the avirulent mutant. Essentially nonfunctional refers to at least about 10- to 1000-fold reduction of the functional activity of the gene product. Optionally, the genetically modified virulence gene can be identified by a change in the physico-chemical characteristics of the gene product, such as a change in molecular weight, isoelectric point, or amino acid composition or the like.

A mutant having a genetically modified virulence gene is also further selected for avirulence and for protection against pasteurellosis, as described herein. In addition, the preferred mutant is selected for long-lasting cross-protective immunity against pasteurellosis as described herein.

In a preferred version, an avirulent transposon (TnphoA) insertion mutant, produced as described herein, can be used to identify and locate a virulence gene of P. multocida. A virulence gene of P. multocida can be identified by Southern blot hybridization with the probe which hybridizes to TnphoA sequences. A virulence gene located on a 9.4 kb EcorV fragment of P. multocida can be mapped by restriction enzymes and sequenced by direct DNA sequencing methods. The virulence gene located on the 9.4 kb EcorV fragment of a virulent P. multocida strain can then be genetically modified by point mutation to generate an avirulent mutant. The mutant is then selected for avirulence and protection against pasteurellosis. The genetic modification of a virulence gene located on the 9.4 kb EcorV fragment can be verified by standard methods, including restriction enzyme mapping or DNA sequencing.

Once produced, an avirulent mutant of P. multocida having at least one genetically modified virulence gene is administered to animals to provide for protection against pasteurellosis. The mutant can be administered by parenteral route, nasal drops, aerosol, and preferably in the drinking water. An effective amount can be determined by injecting different amounts of the mutant into animals and determining the minimum amount that protects against the disease. Preferably, the effective dose is about 10^8 to 10^9 CFU/ml. The mutants of P. multocida can be administered to animals such as cattle, pigs, ducks, turkeys, and chickens. The preferred animal is the turkey.

D. Method for Cloning a P. multocida Virulence Gene and Purifying Recombinantly Produced Virulence Factor

The invention provides a vaccine comprised of a recombinant P. multocida virulence factor encoded in a 9.4 kb EcorV fragment of P. multocida. The recombinant virulence factor can be produced by cloning a gene

encoding a virulence gene into a suitable host by standard methods, as described in Sambrook et al., cited _supra_. The recombinantly produced virulence factor can then be identified and purified from the host cell.

For example, a virulence gene located on a 9.4 kb EcorV fragment, isolated as described herein, can be subcloned into a vector such as the plasmid pBR322. The virulence gene is preferably subcloned at a location in the pBR322 such that it is under the control of the appropriate transcriptional and translational control regions to provide for a high level of gene expression in the host cell. The subcloned virulence gene can be introduced into a suitable host, such as _E. coli_ and expression of the subcloned virulence gene can be monitored by standard methods, including Western blot using an antibody such as pasteurellosis convalescent serum. The recombinant virulence factor can be isolated and purified from _E. coli_ cell lysates by standard methods, including affinity, size exclusion, and/or HPLC chromatography.

The virulence factor can then be tested for the ability to protect against pasteurellosis by immunizing an animal with different amounts of the purified recombinant virulence factor. The immunized animals can be analyzed for the development of protective antibody response by standard methods, including ELISA. The immunized animals are also challenged with at least on virulent serotype of _P. multocida_ to validate whether the virulence factor provides protective immunity against pasteurellosis. The virulence factor of the invention provides for protection against pasteurellosis and preferably long-lasting cross-protective immunity.

EXAMPLE 1

Generation of TnphoA Mutants of Pasteurella multocida

Mutants of Pasteurella multocida were generated by transposon mutagenesis. The transposon utilized was a modified Tn5 (TnphoA) carrying the left insertion sequence of Tn5 linked to the gene for alkaline phosphatase without the natural promoter or signal sequences for the alkaline phosphatase gene. The transposon is present in a plasmid pRT733 which is a pGM703.1 derivative carrying the TnphoA and kanamycin resistance gene and is available from J. Mekalanos, Department of Microbiology and Molecular Genetics, Harvard Medical School. The plasmid pRT733 is a broad host range suicide vector. The plasmid can conjugate with a wide variety of bacteria but is only capable of replicating in those bacterial strains carrying the λ-pir transducing phage. The plasmid cannot replicate without a protein encoded by the λ-pir transducing phage. The alkaline phosphatase gene, when inserted into the bacterial genome along with the transposon, serves as a marker for genes that encode secreted, excreted and membrane bound proteins. The alkaline phosphatase is only active when excreted and has shown to be active as a fusion protein.

The pRT733 plasmid was introduced into a virulent complement resistance streptomycin resistant recipient strain of P. multocida designated Pm-P1059(Sm^R) and mutants containing transpositions were selected in a single step. E. coli K12SM10 lysogenized with λ-pir carrying pRT733 were mated with Pm-P1059(Sm^R) overnight at 37°C on an LB plate. Pm-P1059(Sm^R) insertion mutants were selected on LB plates containing streptomycin (100 μg/ml) and kanamycin (225 μg/ml). Selected colonies were then incubated on LB plates containing the antibiotics and 5-bromo-4-chloro-3-indolyl-phosphate-P-toluidine(XP) (20 μg/ml for 18-24 hours. The XP is a chromogenic substrate for the

alkaline phosphatase enzyme and indicates the presence of secreted alkaline phosphatase by the mutant. Blue colonies were indicative of insertion mutants secreting alkaline phosphatase.

Forty-two TnphoA insertion mutants were isolated. The TnphoA mutants were screened for alkaline phosphatase activity, expression of fusion proteins, expression of iron-regulated outer membrane proteins, loss of complement resistance, and loss of virulence for turkeys. Alkaline phosphatase activity as a fusion protein was measured with the chromogenic substrate XP or P-nitrophenol as described in Taylor et al., J. Bacteriol., 171:1870 (1989). The iron-regulated outer membrane proteins having molecular weights of 94 kDa, 84 kDa, and 76 kDa were detected by standard Western blot methods using antisera specific for these iron-regulated membrane proteins.

Two mutants, designated PmTn-294 and PmTn-396, were positive for alkaline phosphatase activity, expression of fusion proteins and iron-regulated outer membrane proteins. These two mutants were further characterized for virulence in turkeys.

EXAMPLE 2

Identification of Avirulent TnphoA Mutants

The transposition insertion mutants PmTn-294 and PmTn-396 were screened for resistance to complement-mediated lysis and for virulence in turkeys. Resistance to complement mediated lysis correlates with virulence *in vivo*. The parent P-1059 wild strains, Pm-P1059 and Pm-P1059(Sm^R), were resistant to complement-mediated lysis and caused fatal disease in 100% of turkeys within 18 hours.

Both PmTn-294 and PmTn-396 were susceptible to complement mediated lysis. About 1 x 10^8 cells/ml of PmTn-294 and PmTn-396 cells were incubated with 5 ml of turkey plasma containing complement and incubated for

1 hour at 40°C. After incubation, a sample of the PmTn-294 and PmTn-396 cells was serially diluted and plated. After 24 hours of incubation, the number of viable bacteria present after treatment with complement containing turkey plasma was determined by plate counts. Both the PmTn-294 and PmTn-396 showed a 3-fold decrease in viable cells after treatment with complement when compared to the control complement resistant Pm-P1059 strain.

For in vivo virulence testing, groups of five 1-week old turkey poults were inoculated intravenously with 5 x 10^4 colony forming units (CFU) of transposon insertion mutants or the virulent Pm-P1059 strain. The poults were observed for 8 weeks for the presence of disease. All dead turkeys were subjected to postmortem and bacteriological examination to establish the presence of pasteurellosis disease. One hundred percent (100%) of the poults infected with the virulent Pm-P1059 strain died and 100% also showed symptoms of the disease before death. However, infection of poults with either PmTn-294 or PmTn-396 did not result in death or development of the disease. The avirulent mutants are being characterized further to determine the site of the transposon insertion by Southern hybridization.

EXAMPLE 3

Identification of the Location of a Virulence Gene of P. Multocida

To identify the location of a virulence gene inactivated by insertion of the TnphoA transposon and to confirm the presence of the TnphoA transposon in the two insertion mutants, genomic DNA was analyzed by Southern blot hybridization by standard methods.

DNA was obtained from the wild-type virulent P. multocida Pm-P1059, the recipient streptomycin-resistant P. multocida 1059 strain (Pm-P1059 Sm^R), the donor E. coli strain carrying pRT733 (TnphoA

transposon), PmTn-294 (TnphoA insertion mutant), and PmTn-396 (TnphoA insertion mutant) was digested with either KpnI or EcorV. DNA restriction fragments were separated by gel electrophoresis and probed using a EcorI-XhoI digested 1.3 kb fragment or DraI-HpaI digested 7 kb fragment from pRT733. The restriction map of the TnphoA transposon is shown in Fig. 1. The 1.3 kb probe is a EcorI-XhoI fragment having a DNA sequence located between two portions of the left insertion 50 sequence of the TnphoA transposon. The 7 kb probe is a DraI-HpaI probe encoding portions of the left insertion sequence and the right insertion sequence and the kanamycin resistance gene.

The results of the Southern blot hybridization are shown in Fig. 2. DNA from the Pm-P1059, recipient Pm-P1059 Sm^R, and the PmTn-294 digested with KpnI did not hybridize with the 1.3 kb probe. However, DNA digests from the donor E. coli carrying pRT733 and PmTn-396 showed identical fragments which hybridized with the 1.3 kb probe. DNA from the Pm-P1059 and recipient Pm-P1059 Sm^R digested with EcorV also did not hybridize with the 1.3 kb probe. In contrast, DNA from the transconjugant PmTn-396 digested with EcorV showed two bands at 10.9 kb and 9.4 Kb, which hybridized with the 1.3 kb probe. One band at 9.4 kb from PmTn-294 also hybridized with the 1.3 kb probe. The DNA EcorV digest of the pRT733 donor strain showed identical fragments as that of PmTn-396 which hybridized with the probe. The same results were obtained when the digests were probed with the 7 kb DraI-HpaI fragment from pRT733.

The results indicate that avirulence is associated with the insertion of all or a portion of the TnphoA in a 9.4 kb EcorV fragment of the genomic DNA of P. multocida. Virulence gene or genes present in this region and inactivated by this insertion will be mapped by additional restriction enzyme digestion and sequenced

by standard methods, as described in Sambrook et al., cited supra.

All patents and publications cited herein are hereby incorporated by reference. While the present invention has been described in connection with the preferred embodiment thereof, it will be understood many modifications will be readily apparent to those skilled in the art, and this application is intended to cover any adaptations or variations thereof. It is manifestly intended this invention be limited only by the claims and equivalents thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Regents of the University of Minnesota
Morrill Hall
100 Church Street S.E.
Minneapolis, MN 55455
U.S.A.

(ii) TITLE OF INVENTION: COMPOSITION PROTECTIVE AGAINST
P. MULTOCIDA PASTEURELLOSIS INFECTION

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Merchant & Gould
(B) STREET: 3100 Norwest Center
(C) CITY: Minneapolis
(D) STATE: MN
(E) COUNTRY: USA
(F) ZIP: 55402

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/973,070
(B) FILING DATE: 06-NOV-1992
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Woessner, Warren D.
(B) REGISTRATION NUMBER: 30,440
(C) REFERENCE/DOCKET NUMBER: 600.256-WO-01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 612-332-5300
(B) TELEFAX: 612-332-9081

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 64 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vii) IMMEDIATE SOURCE:
(B) CLONE: Termini of insertion sequence 50 of the Tn5 transposon

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CTGACTCTTA TACACAAGTA GCGTCCTGAA CG . . . 32

. . . GCGCAGGGGA TCAAGATCTG ATCAAGAGAC AG 64

WHAT IS CLAIMED IS:

1. A method of immunizing an animal against pasteurellosis comprising:

   producing a stable avirulent immunogenic transposon mediated mutant of P. multocida; and

   administering an effective amount of the stable avirulent mutant to the animal to provide immunity against pasteurellosis.

2. A method according to claim 1, wherein the stable avirulent mutant of P. multocida has the characteristics of P. multocida, ATCC No. 55394.

3. The method according to claim 1, wherein the mutant of P. multocida has the characteristics of P. multocida PmTn-396.

4. The method according to claim 1, wherein the stable avirulent mutant of P. multocida is administered orally.

5. The method of claim 1, wherein in the step of producing a stable avirulent immunogenic transposon mediated mutuant, the transposon mediated mutant is produced by insertion of a transposon selected from the group consisting of Tn1, Tn3, Tn5, TnphoA, Tn7, Tn9, Tn10, and functional fragments thereof.

6. The method of claim 5, wherein in the step of producing a stable avirulent immunogenic transposon mediated mutant, the mutant is produced with a plasmid encoding the left insertion sequence of Tn5 linked to the gene for alkaline phosphatase.

7. The method according to claim 1, wherein the animal is a turkey.

8. A method of immunizing an animal against pasteurellosis, which comprises:

producing a stable, avirulent immunogenic mutant of P. multocida, wherein the mutant has at least one genetically modified virulence gene located in a 9.4 kb EcorV fragment of the P. multocida genome; and

administering an effective amount of the stable avirulent mutant to the animal to provide for immunity against pasteurellosis.

9. A vaccine for protecting an animal against pasteurellosis comprising:

an effective amount of a stable avirulent immunogenic transposon mediated mutant of P. multocida; and

a pharmaceutically acceptable carrier.

10. The vaccine according to claim 9, wherein the stable avirulent mutant has the characteristics of P. multocida ATCC No. 55394.

11. The vaccine according to claim 9, wherein the pharmaceutically acceptable carrier is water.

12. An avirulent immunogenic mutant of P. multocida having the characteristics of ATCC No. 55395.

13. A vaccine for protecting an animal against pasteurellosis, which comprises:

an effective amount of a stable avirulent immunogenic mutant of P. multocida, wherein the mutant has at least one genetically modified virulence gene located in a 9.4 kb EcorV fragment of the P. multocida genome; and

a pharmaceutically acceptable carrier.

14. A vaccine for protecting an animal against pasteurellosis, which comprises:

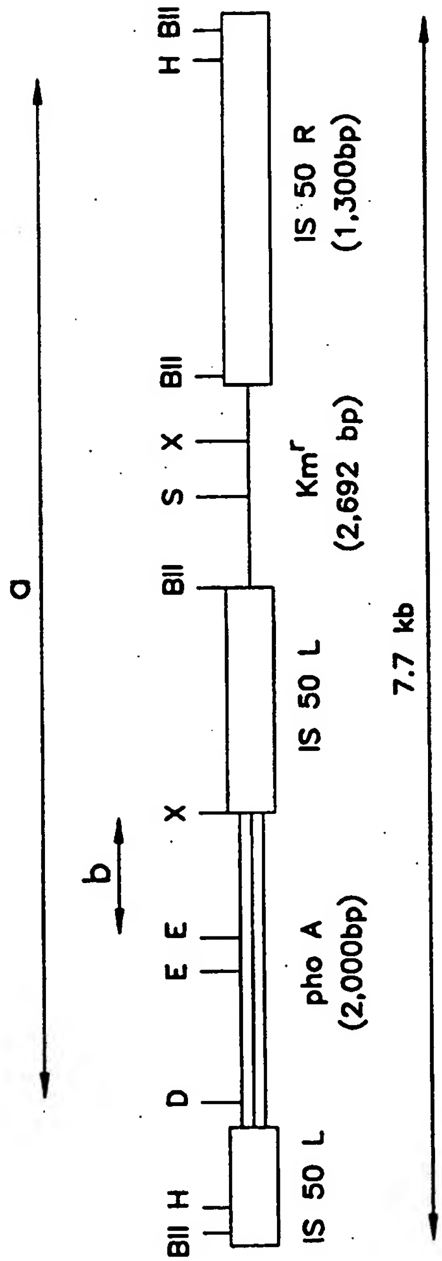
an effective amount of a recombinantly produced virulence factor from P. multocida; and

a pharmaceutically acceptable carrier.

15. A vaccine for protecting an animal against pasteurellosis, which comprises:

an effective amount of a recombinantly produced virulence factor from P. multocida, wherein the virulence factor is encoded by a 9.4 kb EcorV fragment of the P. multocida genome.

RESTRICTION MAP OF TnphoA

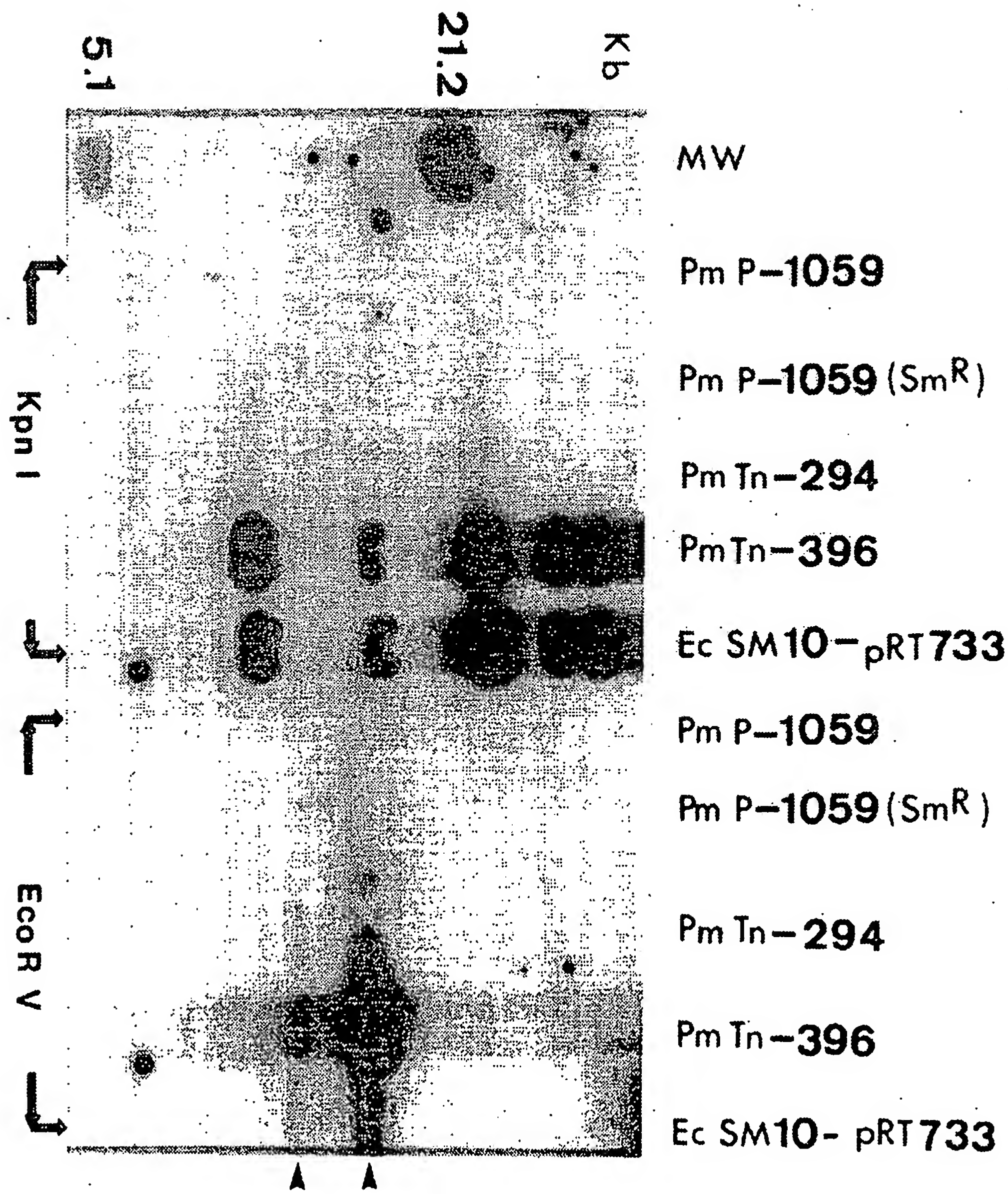


a: 7kb PROBE DIGESTED WITH Dra I–Hpa I
b: 1.3 kb PROBE DIGESTED WITH EcoR I–Xho I

BII:Bgl II, H:Hpa I, D:Dra I, E:EcoR I, X:Xho I, S:Sma I

FIG. 1

FIG. 2


5.1
21.2
Kb
MW
Kpn I
Pm P-1059
Pm P-1059 (Sm^R)
Pm Tn-294
Pm Tn-396
Ec SM10-pRT733
EcoR V
Pm P-1059
Pm P-1059 (Sm^R)
Pm Tn-294
Pm Tn-396
Ec SM10- pRT733

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/10600

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/102; C07K 15/04; C12N 15/31
US CL :424/88, 92, 93D; 435/72.3, 252.3; 530/350, 825
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/88, 92, 93D; 435/72.3, 252.3; 530/350, 825

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, DERWENT
search terms: multocida, toxin, virulence factor, vaccine

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US, A, 4,999,191 (Glisson et al.) 12 March 1991, see entire document.	8,12,13 ----------- 1-7,9-11
X -- Y	US, A, 4,293,545 (Kucera et al.) 06 October 1981, see entire document.	8,12,13 ----------- 1-7,9-11
X -- Y	US, A, 4,169,886 (Hertman et al.) 02 October 1979, see entire document.	8,12,13 ----------- 1-7,9-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
03 February 1994

Date of mailing of the international search report
FEB 14 1994

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. NOT APPLICABLE

Authorized officer
Michael S. Tuscan Ph.D. D. Kryza for
Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	Poultry Science, Volume 54, issued 1974, K.L. Heddleston et al., "Fowl Cholera: Immunologic and Serologic Response in Turkeys to Live Pasteurella multocida Vaccine in the Drinking Water", pages 217-221, see entire document.	8,12,13 ---------- 1-7,9-11
X -- Y	Poultry Science, Volume 51, issued 1972, B.W. Bierer et al., "Immunologic Response of Turkeys to an Avirulent Pasteurella multocida Vaccine in the Drinking Water", pages 408-416, see entire document.	8,12,13 ---------- 1-7,9-11
Y	US, A, 4,735,801 (Stocker) 05 April 1988, see entire document.	1-7,9-11
Y	Journal of General Microbiology, Volume 135, issued 1989, A. Ndubisi et al., "Transfer and Properties of Some Natural and Suicide Replicons in Pasteurella multocida", pages 3345-3352, see entire document.	1-7,9-11
Y	Proceedings of the National Academy of Sciences USA, Volume 82, issued December 1985, C. Manoil et al., "TnphoA: A Transposon Probe for Protein Export Signals", pages 8129-8133, see entire document.	1-7,9-11
X	Infection and Immunity, Volume 59, Number 4, issued April 1991, S.K. Petersen et al., "Recombinant Derivatives of Pasteurella multocida Toxin: Candidates for a Vaccine against Progressive Atrophic Rhinitis", pages 1387-1393, see entire document.	14,15